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Advances in Brief

Growth Suppression of Human Head and Neck Cancer Cells by the Introduction f a Wild-Type *p53* Gene via a Recombinant Adenovirus[1]

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Abstract

Mutations of the *p53* gene constitute one of the most frequent genetic alterations in squamous cell carcinoma of the head and neck (SCCHN). In this study, we introduced wild-type *p53* into two separate SCCHN cell lines via a recombinant adenoviral vector, Ad5CMV-p53. Northern blotting showed that following infection by the wild-type *p53* adenovirus (Ad5CMV-p53), cells produced up to 10-fold higher levels of exogenous *p53* mRNA than cells treated with vector only (without *p53*). Western blotting showed that the increased levels of p53 protein produced in the Ad5CMV-p53-infected cells were a reflection of *p53* mRNA expression. *In vitro* growth assays revealed growth arrest following Ad5CMV-p53 infection as well as cell morphological changes consistent with apoptosis. *In vivo* studies in nude mice with established s.c. squamous carcinoma nodules showed that tumor volumes were significantly reduced in mice that received peritumoral infiltration of Ad5CMV-p53. These data suggest that Ad5CMV-p53 may be further developed as a potential novel therapeutic agent for SCCHN since introduction of wild-type p53 into SCCHN cell lines attenuates their replication and tumor growth.

Introduction

Patients with SCCHN[3] are afflicted with a disease process that often has profound effects on speech, swallowing, and cosmesis. Furthermore, the overall rate of survival among these patients has remained unchanged at approximately 45% for nearly 30 years since contemporary surgery and radiation therapy were instituted (1). Treatment failures among these patients remain local and regional; only 10–15% of patients with the disease die of distant metastasis alone (2).

Although we have gained in understanding of the molecular events in the initiation and progression of SCCHN, they continue to require intensive investigation. A recent study identifying loss of heterozygosity of chromosome 9p21–22 as the most frequent genetic alteration in SCCHN suggested that this may be an early event in progression toward this neoplasm (3). Additionally, amplification and/or overexpression of cellular and nuclear oncogenes, such as c-*erB*-1 (4), *int*-2 (5), *bcl*-1 (6) and c-*myc* (7), have been documented in these cancers. The tumor suppressor gene *p53* has been the subject of immense interest and investigation in recent years. Alterations in the *p53* gene, including deletion, insertion, and point mutation, are the most frequent genetic events in many different carcinomas, such as those of the colon (8), breast (9), and lung (10), as well as soft-tissue sarcomas and leukemias (11). Several investigators have demonstrated the high frequency of *p53* gene alterations in SCCHN (12, 13).

There is considerable evidence implicating mutations of the *p53* gene in the etiology of many human cancers (14). Reports have demonstrated that growth of several different human cancer cell lines, including representatives of colon cancer (15), glioblastoma (16), breast cancer (17), and osteosarcoma (18), can be functionally suppressed by DNA transfection or retrovirus-mediated transfer of the wild-type *p53* gene. This gene may have an important role not only in cell growth but in apoptosis (programmed cell death). Induction of exogenous expression of wild-type *p53* has been shown to induce apoptosis in colon cancer cell lines (19) and in human lung cancer spheroids (20).

The adenoviral vector has emerged as a leading candidate for *in vivo* gene therapy in the past few years. It enjoys an advantage over traditional DNA transfection and retroviral transfer in its high efficiency of transferring potentially therapeutic genes into a wide range of host cells (21). The recently created adenoviral vector containing wild-type *p53* (Ad5CMV-p53; Ref. 22) provides us with an attractive delivery system to investigate the effect of exogenous wild-type *p53* on SCCHN cell lines both *in vitro* and *in vivo*. The outcome of this study indicates that further development of the p53 adenovirus or other novel molecular therapies for SCCHN is warranted.

Materials and Methods

Cell Lines and Culture Conditions. Human SCCHN cell lines Tu-138 and Tu-177 were both established at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center. Tu-138 and Tu-177 were established from a gingivo-labial moderately differentiated squamous carcinoma and a poorly differentiated squamous carcinoma of the larynx, respectively. Both cell lines were developed via primary explant technique and are cytokeratin positive and tumorigenic in athymic nude and SCID mice. These cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated FBS with penicillin/streptomycin.

Recombinant Adenovirus Preparation and Infection. The recombinant p53 adenovirus (Ad5CMV-p53; Ref. 22) contains the CMV promoter, wild-type p53 cDNA, and SV40 polyadenylation signal in a minigene cassette inserted into the E1-deleted region of modified Ad5. Viral stocks were propagated in 293 cells. Cells were harvested 36–40 h after infection, pelleted, resuspended in phosphate-buffered saline, and lysed; cell debris was removed by subjecting the cells to CsCl gradient purification. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. Infection was carried out by the addition of the virus to the DMEM/F12 medium and 2% FBS to the cell monolayers. The cells were incubated at 37°C for 60 min with constant agitation. Then complete medium (DMEM/F12–10% FBS) was added, and the cells were incubated at 37°C for the desired length of time.

Northern Blot Analysis. Total RNA was isolated by the acid-guanidinium thiocyanate method of Chomczynski and Sacchi (23). Northern analyses were performed on 20 μg of total RNA. The membrane was hybridized with a *p53*

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[3] The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F-12 medium; FBS, fetal bovine serum; Ad5, adenovirus 5; CMV, cytomegalovirus; Ad5CMV-p53, wild-type p53 adenovirus; cDNA, complementary DNA; MOI, multiplicity of infection; SDS, sodium dodecyl sulfate; β-gal, β-galactosidase; dl312, replication-defective adenovirus; PFU, plaque forming units.

cDNA probe labeled by the random primer method in 5 × SSC-5 × Denhardt's solution-0.5% SDS-denatured salmon sperm DNA (20 μg/ml). The membrane was also stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA for RNA loading control. The relative quantities of *p53* expressed were determined by densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Western Blot Analysis. Total cell lysates were prepared by sonicating the cells 24-h postinfection in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) for 5 s. Fifty μg of protein from samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond-ECL membrane (Amersham). The membrane was blocked with Blotto/Tween (5% nonfat dry milk, 0.2% Tween 20, and 0.02% sodium azide in phosphate-buffered saline) and probed with the primary antibodies, mouse anti-human p53 monoclonal antibody PAb1801 and mouse anti-human β-actin monoclonal antibody (Amersham), and the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN). The membrane was processed and developed as the manufacturer suggested.

Immunohistochemical Analysis. The infected cell monolayers were fixed with 3.8% formalin and treated with 3% H_2O_2 in methanol for 5 min. Immunohistochemical staining was performed by using the Vectastain Elite kit (Vector, Burlingame, CA). The primary antibody used was the anti-p53 antibody PAb1801, and the secondary antibody was an avidin-labeled anti-mouse IgG (Vector). The biotinylated horseradish peroxidase avidin-biotin complex reagent was used to detect the antigen-antibody complex. Preadsorption controls were used in each immunostaining experiment. The cells were then counterstained with Harris hematoxylin (Sigma Chemical Co., St. Louis, MO).

Cell Growth Assay. Cells were plated at a density of 2 × 10^4 cells/ml in 6-well plates in triplicate. Cells were infected with either wild-type (Ad5CMV-p53) or replication-deficient adenovirus as a control. Cells were harvested every 2 days and counted; their viability was determined by trypan blue exclusion.

Inhibition of Tumor Growth *in Vivo*. The effect of Ad5CMV-p53 on established s.c. tumor nodules was determined in nude mice in a defined pathogen-free environment. Experiments were reviewed and approved by institutional committees for both animal care and use and for recombinant DNA research. Briefly, following induction of acepromazine/ketamine anesthesia, three separate s.c. flaps were elevated on each animal, and 5 × 10^6 cells in 150 μl of complete media were injected s.c. into each flap using a blunt needle; the cells were kept in the pocket with a horizontal mattress suture. Four animals were used for each cell line. After 4 days, the animals were reanesthetized, and the flaps were reelevated for the delivery of 100 μl of: (*a*) Ad5CMV-p53 (10^8 PFU) in the right anterior flap; (*b*) replication-defective virus (10^8 PFU) in the right posterior flap; and (*c*) transport medium alone, in the left posterior flank. All injection sites had developed s.c. visual and palpable nodules before treatment was administered. Animals were observed daily and sacrificed on day 20. *In vivo* tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters. Following sacrifice, excised tumors were measured three dimensionally by microcalipers to determine tumor volume. A nonparametric Friedman's two-way analysis of variance test was used to test the significance of the difference between means of samples; the SPSS/PC+ software package (SPSS, Inc., Chicago, IL) was used.

Results

Adenoviral Infection of SCCHN Cells. The conditions for optimal adenoviral transduction of Tu-138 and Tu-177 cells were determined by infecting these cells with adenovirus expressing the *Escherichia coli* β-gal gene. The transduction efficiency was assessed by counting the number of blue cells after X-gal staining. There appeared to be a linear relationship between the number of infected cells and the number of adenovirus particles used. Cells inoculated with a single dose of 100 MOI β-gal adenovirus exhibited 60% blue cells (Fig. 1*A*), and this was improved to 100% by multiple infections (data not shown). The transduction efficiency of this vector in SCCHN cells is quite different from that of other cell lines examined previously; HeLa, HepG2, LM2, and human non-small cell lung cancer cell lines

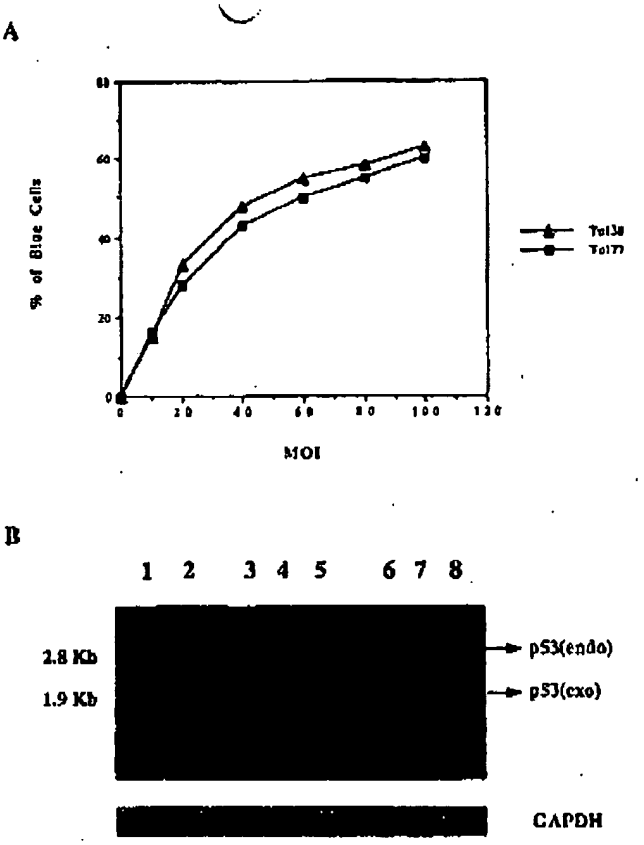

Fig. 1. *A*, transduction efficiency of SCCHN cell lines Tu-138 (▲) and Tu177 (■). A recombinant β-gal adenovirus was used to infect the cells at different MOIs ranging from 10 to 100. The percentages of β-gal-positive cells were obtained from scoring 500 cells each on replicate dishes. *B*, expression of exogenous *p53* mRNA 24 h after Ad5CMV-p53 infection. *Lanes 1* and *2*, 293 and K562 cells, respectively. *Lanes 3* and *6*, mock-infected Tu-138 and Tu-177 cells. *Lanes 4* and *7*, Tu-138 and Tu-177 cells infected with dl312. *Lanes 5* and *8*, Tu-138 and Tu-177 cells infected with Ad5CMV-p53.

showed 97 to 100% infection efficiencies after incubation with 30 to 50 MOI β-gal adenovirus (22).

Expression of Exogenous *p53* mRNA in Adenovirus-infected SCCHN Cells. Two human SCCHN cell lines were chosen for this study; both cell lines Tu-138 and Tu-177 possess a mutated *p53* gene (unpublished data). The recently created recombinant wild-type p53 adenovirus, Ad5CMV-p53, was used to infect Tu-138 and Tu-177 cells. Twenty-four h after infection, total RNA was isolated, and Northern blot analysis was performed. The transformed primary human embryonal kidney cell line 293 was used as a positive control because of its high level of expression of the *p53* gene product, whereas K562, a lymphoblastoma cell line with a homozygous deletion of the *p53* gene, was the negative control (Fig. 1*B*, *Lanes 1* and *2*, respectively). Due to unequal loading, only a fraction of the endogenous *p53* mRNA was detected in the 293 cells (Fig. 1*B*, *bottom panel*). The levels of the 2.8-kilobase endogenous *p53* mRNA detected in the samples isolated from mock-infected cells (Fig. 1*B*, *Lanes 3* and *6*) and from the cells infected with a replication-defective adenovirus, dl312 (Fig. 1*B*, *Lanes 4* and *7*), were similar. Up to 10-fold higher levels of exogenous 1.9-kilobase *p53* mRNA were present in the cells infected with Ad5CMV-p53 (Fig. 1*B*, *Lanes 5* and *8*), indicating that the exogenous *p53* cDNA was successfully transduced into these cells and efficiently transcribed. Interestingly, the level of endogenous *p53* mRNA in these cells was 5-fold higher than in the experimental controls. Northern blots exhibited no evidence of Ad5CMV-p53 (DNA) contamination of RNA.

Expression of p53 Protein in Adenovirus-infected SCCHN Cells. Western blot analysis was performed to compare the levels of *p53* mRNA to the amount of p53 protein produced. A p53 band, recognized by monospecific anti-p53 antibody, PAb1801, was observed in cellular extracts isolated from all samples except K562 cells (Fig. 2*A*, *Lane 8*). Cell line 293 showed high levels of p53 protein (Fig. 2*A*, *Lane 1*). Samples isolated from mock-infected Tu-138 and Tu-177 cells exhibited low levels of p53 protein (Fig. 2*A*, *Lanes 2* and *5*). The level of p53 expression remained similar in those cells infected with the dl312 adenovirus (Fig. 2*A*, *Lanes 3* and *6*). The levels of p53 antigen detected in Ad5CMV-p53-infected cells were significantly higher than the levels of the endogenous mutated proteins in both cell lines (Fig. 2*A*, *Lanes 5* and *7*). This result indicates that the exogenous *p53* mRNA produced from cells infected with Ad5CMV-p53 is efficiently translated into immunoreactive p53 protein. Furthermore, immunohistochemical analysis of cells infected with Ad5CMV-p53 revealed the characteristic nuclear staining of p53 protein (Fig. 2*B*, *right panel*), whereas mock-infected cells failed to show similar staining despite the presence of the p53 protein in these cells (Fig. 2*B*, *left panel*). This inability to detect the protein may be attributable to the insensitivity of the assay.

Effect of Exogenous p53 on SCCHN Cell Growth *in Vitro*. Cells infected with control virus dl312 had growth rates similar to those of the mock-infected cells (Fig. 3), whereas growth of the Ad5CMV-

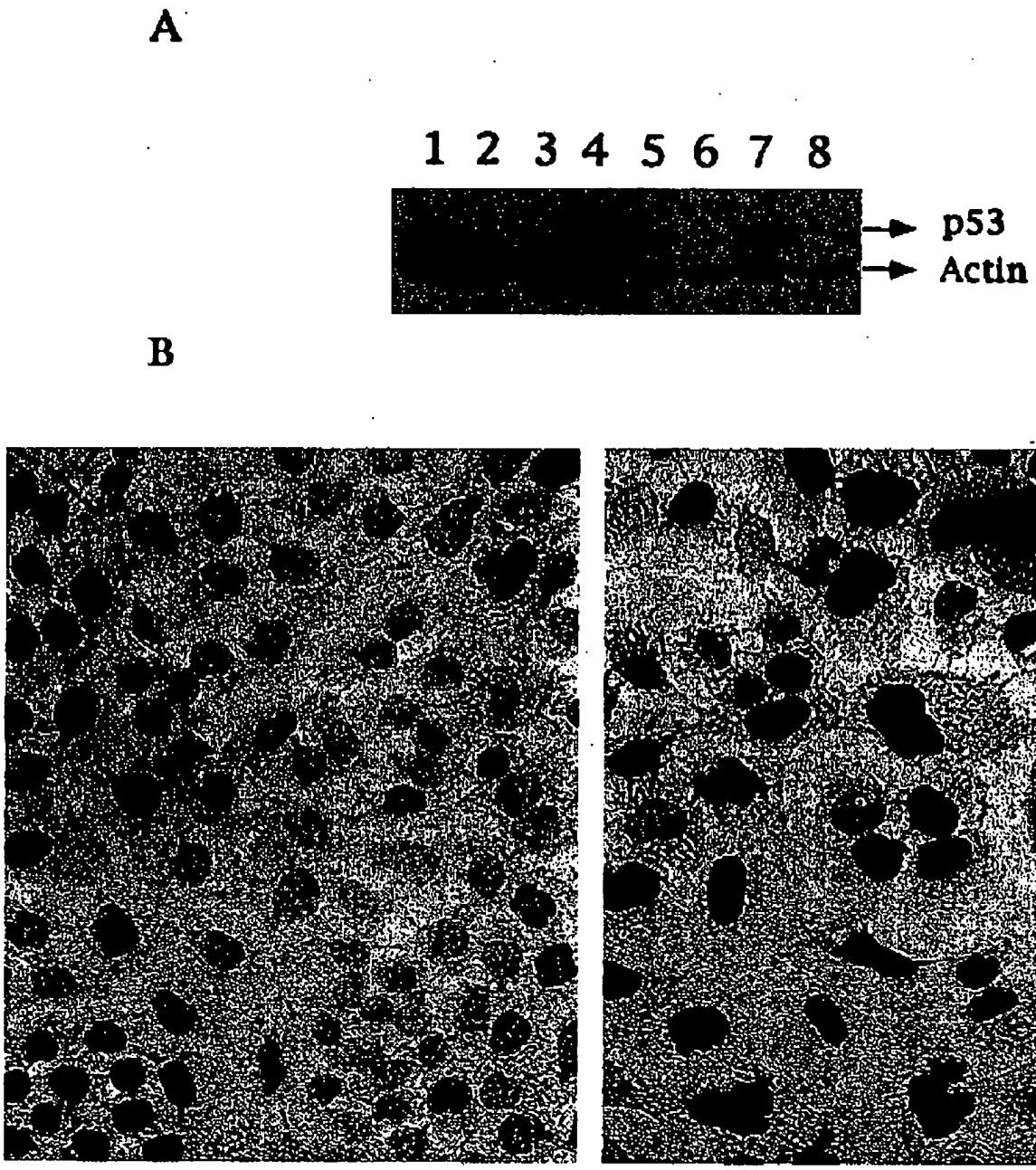

Fig. 2. *A*, Western blot analysis. Cellular extracts isolated from cells 24 h postinfection were subjected to SDS-polyacrylamide gel electrophoresis. *Lanes 1* and *8*, 293 and K562 cells, respectively. *Lanes 2* and *5*, mock-infected Tu-138 and Tu-177 cells. *Lanes 3* and *6*, Tu-138 and Tu-177 cells infected with dl312. *Lanes 4* and *7*, Tu-138 and Tu-177 cells infected with the Ad5CMV-p53. *B*, representative immunohistochemical staining of mock-infected Tu-138 cells (*left*) and Ad5CMV-p53-infected Tu-138 cells (*right*) 24-h postinfection. × 250.

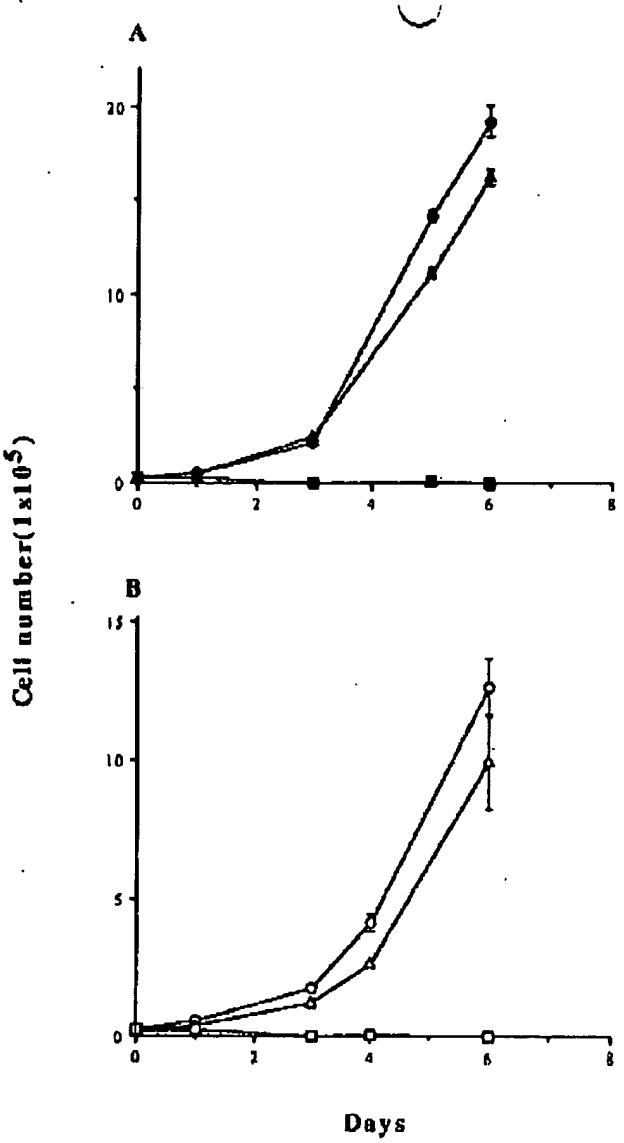

Fig. 3. Inhibition of SCCHN cell growth *in vitro*. *A*, growth curve of mock-infected Tu-138 cells (●), dl312-infected cells (▲), and Ad5CMV-p53-infected cells (■). *B*, growth curve of mock-infected Tu-177 cells (○), dl312-infected cells (△), and Ad5CMV-p53-infected cells (□). At each indicated time point, three dishes of cells were trypsinized and counted. The mean of cell counts per triplicate wells following infection were plotted against the number of days since infection; *bars*, SEM.

p53-infected Tu-138 (Fig. 3*A*) and Tu-177 (Fig. 3*B*) cells was greatly suppressed. Twenty-four h after infection, an apparent morphological change occurred with portions of the cell population rounding up and their outer membranes forming blebs. These are part of a series of histologically predictable events that constitute programmed cell death. The effect was more prominent for Tu-138 than for Tu-177 cells. Cells infected with the replication-defective adenovirus, dl312, demonstrated normal growth characteristics with no histomorphological abnormalities. Growth assays were reproducible in four repeated experiments.

Inhibition of Tumor Growth *in Vivo*. Seven animals were tested for each cell line. One animal in the Tu-177 group died following the second flap surgery and delivery of the therapeutic interventions, presumably due to profound anesthesia and subsequent mutilation by cage mates. Necropsy revealed no evidence of metastasis or systemic effects. Fig. 4 shows representative Tu-138 (*left*) and Tu-177 recipients (*right*). Sizable tumors are apparent on both posterior flaps of the animals (*i.e.*, the sites that did not receive Ad5CMV-p53). The lack of tumor progression is significant in the right anterior flaps of the animals which received Ad5CMV-p53 ($P < .04$). That Tu-177 cells have a slower growth rate has been established previously in these animals.[4] Two animals in the Tu-177 group had complete clinical and pathological regression of their established s.c. tumor nodule. Two animals in the Tu-138 group were killed early because they were experiencing rapid growth and ulceration of the control tumor sites. All surgical sites had developed lesions of at least 6 mm^3 before intervention. The tumor volumes on necropsy are shown in Table 1.

Discussion

Mutations or deletions of the *p53* tumor suppressor gene are the most frequent genetic alterations reported in SCCHN. Since the wild-type *p53* gene is believed to be involved primarily in delivering antiproliferative signals that may be capable of antagonizing the growth-stimulatory signals propagated by oncogene products, the potential molecular therapeutic effect of this gene in SCCHN deserves attention.

The rapid development in the field of gene therapy, including the creation of adenoviral vectors, has created an environment that is well suited for progress toward novel gene therapy of SCCHN. Because of their natural tropism for aerodigestive tract epithelium, adenoviruses may be uniquely suitable for the transient delivery of genes to cancers in these epithelial tissues. The recombinant, replication-defective adenoviruses that have been developed for gene therapy are missing the entire E1a and part of the E1b regions and are, therefore, capable of propagating only in cells that can provide the E1 proteins in trans, such as the 293 cell line. In the past few years, recombinant adenoviruses have been extensively developed and used for *in vivo* gene therapy. The high transfer efficiency of adenoviral vectors over a broad range of hosts both *in vitro* and *in vivo* make them attractive vehicles for molecular therapy. Recently, a recombinant wild-type p53 adenoviral vector (Ad5CMV-p53) was generated. This provided us with an excellent candidate for investigation of the biological effects of the wild-type *p53* gene product on SCCHN cells bearing the mutated *p53* gene. Using a β-gal recombinant adenovirus, the gene transfer efficiency of SCCHN cells was established. Approximately 60% of SCCHN cells were positive after X-gal staining. There appeared to be a linear correlation between the number of cells expressing the gene and the amount of viral particles used in the experiment. This result coincided with the efficiency obtained in cells infected with Ad5CMV-p53 after immunostaining by using a monoclonal anti-p53 antibody. Our observed transduction efficiency was lower than that achieved in other cell lines tested, including HeLa, HepG2, LM2, and the human non-small cell lung cancer cell lines. This discrepancy could be due to a host of factors, including receptor variations and differences in membrane characteristics among the cell lines. Additionally, the transduction efficiency of SCCHN cells may have been underestimated by limitations of light microscopic analyses.

Ad5CMV-p53 mediated a high level of expression of the *p53* gene in SCCHN cells. Two *p53* mRNA species were detected in the Ad5CMV-p53-infected cells. The high level of 1.9-kilobase mRNA was derived from the transduced *p53* cDNA following infection with Ad5CMV-p53, indicating that the adenoviral vector is an efficient vehicle for gene delivery into human SCCHN cells. Moreover, the levels of endogenous 2.8-kilobase mRNA were higher in the transduced cells than in the controls, presumably due to the effect of wild-type *p53* gene product. This phenomenon of transcriptional

[4] Unpublished data.

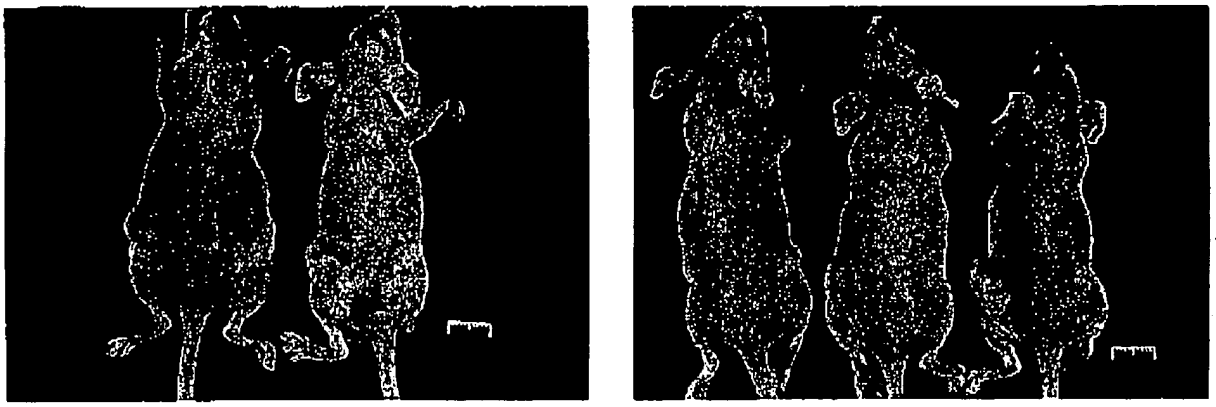

Fig. 4. Inhibition of SCCHN cell growth *in vivo*. Pictures of the representative nude mice studies for both Tu-138 (*left*) and Tu-177 (*right*) cell lines 20 days following therapeutic interventions. The right posterior flank received dl312, the left flank received transport medium alone, and the right anterior flap received Ad5CMV-p53, all 4 days following the establishment of a s.c. tumor.

Table 1 *Effect of Ad5CMV-p53 on tumor growth in nude mice*[a]

Treatment	Mean volume (mm^3 ± SEM)	
	Tu-138 (7)	Tu-177 (6)
Ad5CMV-p53	22.3 ± 14	13 ± 18
Ad5(dl312)	803 ± 300	533 ± 148
Medium	1297 ± 511	421 ± 143
Significance	*P*	*P*
p53[b]:dl312	0.03	0.02
p53:medium	0.04	0.03

[a] The cells were injected s.c. at 5×10^6 cells/flap. Tumor sizes were determined at day 20 after treatment. Numbers in parentheses, the number of animals evaluated.
[b] Ad5CMV-p53 is abbreviated as p53; dl312 is an abbreviation for Ad5(dl312).

autoregulation of the *p53* gene has been well documented in murine cell lines in which the wild-type *p53* can transactivate its own promoter and the mutant *p53* fails to regulate the *p53* promoter (24).

Due to the episomal property of adenoviral vectors, all the input DNA following infection with Ad5CMV-p53 is presumably degraded slowly throughout incubation. By using polymerase chain reaction-based detection techniques, DNA can be detected as late as 14 days postinfection (data not shown).

Western blot analysis demonstrated that there were few or no changes of p53 protein levels between mock- and replication-defective adenovirus-infected cells, whereas production of p53 protein was significant in Ad5CMV-p53-infected cells, suggesting that the exogenous *p53* mRNA was efficiently translated. Time course protein expression studies have shown protein expression to peak 3 days postinfection and progressively decline to still detectable Western blotting levels on day 15 (22). Functionally, these SCCHN cells transduced with wild-type p53 gene exhibited significant inhibition of growth *in vitro* as compared to the mock-infected and replication-defective cells, thus clearly illustrating that these results were not mediated by the virus itself. The mechanism by which wild-type p53 protein inhibits growth *in vitro* may be related to arrest of the G_1 cell cycle (18), apoptosis (19, 20), or induction of another tumor suppressor gene such as *WAF1/CIP1* (25). The induction of apoptosis is one of the several documented functions of wild-type *p53*. When Tu-138 and Tu-177 cells were infected with Ad5CMV-p53 at 100 plaque-forming units/cell, the characteristic apoptotic histomorphology, such as rounded-up cells and the formation of blebs, was apparent as early as 4 h after infection and was followed rapidly by cell death (data not shown). However, the mechanism of growth suppression and cell death induced by Ad5CMV-p53 requires further investigation.

Encouraging results were also obtained in the nude mice studies. Tumor growth in the Ad5CMV-p53-infected cells was suppressed by at least 60 times more than in the experimental controls. These *in vivo* results confirmed the *in vitro* effects of Ad5CMV-p53 on human SCCHN cells, suggesting that the wild-type p53 protein mediates a potentially therapeutic effect. Although the *in vivo* studies are in their infancy, they nevertheless portend the development of a model for gene therapy in SCCHN that uses p53 adenovirus as a therapeutic intervention. Information derived from such studies could be expanded in the development of other novel molecular therapies that use adenoviral vectors, not only in SCCHN but in other human cancers. Several critical questions remain unanswered. How should the insult from antibodies that may arise in animals or patients following viral treatment be alleviated? How safe is this virus in humans? The results of the preliminary studies justify further investigation of *in vivo* animal models as well as mechanisms through which wild-type *p53* regulates these *in vitro* and *in vivo* effects.

References

1. Cancer Facts and Figures. Publication No. 90-425, M. No. 5008-LE. Washington, DC: American Cancer Society, 1990.
2. Thawley, S. E., and Panje, W. R. (eds.). Comprehensive Management of Head and Neck Tumors, Vol. 2, pp. 1158-1172. Philadelphia: W. B. Saunders, 1987.
3. van der Riet, P., Nawroz, H., Hruban, R. H., Corio, R., Tokino, K., Koch, W., and Sidransky, D. Frequent loss of chromosome 9 p21-22 early in head and neck cancer progression. Cancer Res., *54:* 1156-1158, 1994.
4. Yamamoto, T., Kamata, N., Kawano, H., Shimizu, S., Kuroki, T., Toyoshima, K., Rikimaru, K., Nomura, N., Ishizaki, R., Pastan, I., Gamou, S., and Shimizu, N. High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. Cancer Res., *46:* 414-416, 1986.
5. Somers, K. D., Cartwright, S. L., and Schechter, G. L. Amplification of the *int-2* gene in human head and neck squamous cell carcinomas. Oncogene, *5:* 915-920, 1990.
6. Berenson, R., Yung, J., and Mickel, R. A. Frequent amplification of the *bcl-1* locus in head and neck squamous cell carcinomas. Oncogene, *4:* 1111-1116, 1989.
7. Field, J. K., Spandidos, D. A., Stell, P. M., Vaughan, E. D., Evan, G. I., and Moore, J. P. Elevated expression of the c-myc oncoprotein correlates with poor prognosis in head and neck squamous cell carcinoma. Oncogene, *4:* 1463-1468, 1989.
8. Rodrigues, N. R., Rowan, A., Smith, M. E. F., Kerr, I. B., Bodmer, W. F., Gannon, J. V., and Lane, D. P. p53 mutations in colorectal cancer. Proc. Natl. Acad. Sci. USA, *87:* 7555-7559, 1990.
9. Bartek, J., Iggo, R., Gannon, J., and Lane, D. P. Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene, *5:* 893-899, 1990.
10. Takahashi, T., Takahashi, T., Suzuki, H., Hida, T., Sekido, Y., Ariyoshi, Y., and Ueda, R. The *p53* gene is very frequently mutated in small-cell lung cancer with a distinct nucleotide substitution pattern. Cancer Res., *52:* 734-736, 1992.
11. Mashal, R., Sbuhrid, M., Tulpez, M., Kantarjian, H., Smith, L., Beran, M., Cork, A., Trujillo, J., Gutterman, J., and Deisseroth, A. Rearrangement and expression of p53

in the chronic phase and blast crisis of chronic myelogenous leukemia. Blood, *75:* 180–189, 1990.

12. Maestro, R., Dolcetti, R., Gasparotto, D., Doglioni, C., Pelucchi, S., Barzan, L., Grandi, E., and Boiocchi, M. High frequency of *p53* gene alterations associated with protein overexpression in human squamous cell carcinoma of the larynx. Oncogene, *7:* 1159–1166, 1992.
13. Chung, K. Y., Mukhopadhyay, T., Kim, J., Casson, A., Ro, J. Y., Goepfert, H., Hong, W. K., and Roth, J. A. Discordant *p53* gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. *53:* 1676–1683, 1993.
14. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. p53 mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.
15. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K., and Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science (Washington DC), *249:* 912–915, 1990.
16. Mercer, W. E., Shields, M. T., Amin, M., Sauve, G. J., Appella, E., Romano, J. W., and Ullrich, S. J. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc. Natl. Acad. Sci. USA, *87:* 6166–6170, 1990.
17. Cai, D. W., Mukhopadhyay, T., Liu, Y., Fujiwara, T., and Roth, J. A. Stable expression of the wild-type *p53* gene in human lung cancer cells after retrovirus-mediated gene transfer. Hum. Gen. Ther., *4:* 617–624, 1993.
18. Diller, L., Kassel, J., Nelson, C. E., Gryka, M. A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S. J., and Vogelstein, B. p53 functions as a cell cycle control protein in osteosarcomas. Mol. Cell. Biol., *10:* 5772–5781, 1990.
19. Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B., and Costa, J. Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc. Natl. Acad. Sci. USA, *89:* 4495–4499, 1992.
20. Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Cai, D. W., Owen-Schaub, L. B., and Roth, J. A. A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. Cancer Res., *53:* 4129–4133, 1993.
21. Kozarsky, K. F., and Wilson, J. M. Gene therapy: adenovirus vectors. Curr. Opin. Gen. Dev., *3:* 499–503, 1993.
22. Zhang, W-W., Fang, X., Mazur, W., French, B. A., Georges, R. N., and Roth, J. A. High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. Cancer Gen. Ther., *1:* 1–10, 1994.
23. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.
24. Deffie, A., Wu, H., Reinke, V., and Lozano, G. The tumor suppressor p53 regulates its own transcription. Mol. Cell. Biol., *13:* 3415–3423, 1993.
25. El-Deiry, W. S., Harper, J. W., O'Connor, P. M., *et al.* WAF1/CIP1 is induced in p53-mediated G_1 arrest and apoptosis. Cancer Res., *54:* 1169–1174, 1994.